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14. ABSTRACT

Blood cell production is a highly dynamic process, designed to respond to stresses such as infection or bleeding over the entire lifespan of a person or animal. During normal conditions, there is a careful balance between blood cells made and blood cells removed due to aging or damage. A highly regulated process called programmed cell death removes cells, and bone marrow failure occurs when more cells are removed than can be replaced. There are two major pathways of programmed cell death: apoptosis and necrosis. Simply described, apoptotic cells implode in an immune silent process, and necrotic cells explode, activating an inflammatory response. In bone marrow failure disorders, necrotic death of bone marrow cells increases normal bone marrow damage by amplifying the pathological inflammatory response. We hypothesize that increased necrotic cell death initiates a feed forward inflammatory process that kills normal bone marrow, and that targeting this programmed necrotic cell death could be used for therapeutic benefit. This proposal is focused on 1) determining how necrotic cell death impacts the surrounding normal bone marrow, and 2) determining whether inhibiting necrosis can rescue bone marrow failure in mouse models of MDS.

15. SUBJECT TERMS

Nothing listed

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1. Introduction

Normal bone marrow function requires stringent control of production of new cells (proliferation) and removal of aging or damaged cells (programmed cell death). Patients with bone marrow failure disorders such as Myelodysplastic syndrome (MDS) have increased bone marrow programmed cell death, and increased levels of death-inducing cytokines such as TNFα. Discoveries in the last several years have demonstrated that in addition to apoptosis, TNFα also activates a novel form of cell death, programmed necrosis. Apoptotic cells implode in caspase-driven and immune silent process, whereas necrotic cells explode in a Rip kinase-driven process, releasing cellular contents (DAMPS) and eliciting an immune response. We find increased Rip1 kinase expression in 70% of MDS patient samples tested suggesting that necroptosis is activated in MDS. We also find that bone marrow from mouse models harboring known genetic mutations found in MDS (Asxl1-/-, Asxl1-/-Tet2-/-) display increased Rip1 kinase, suggesting that MDS genetic/epigenetic alterations result in increased necroptosis signaling that contributes to bone marrow cell death.

Substantial data demonstrate that MDS is a clonal stem cell disorder(Graubert et al., 2012; Walter et al., 2011; Walter et al., 2012; Walter et al., 2013). Recent data has also identified a rare population of bone marrow cells as the MDS-propagating population in patients with low to intermediate-risk MDS, and these MDS-propagating cells are expanded, representing 94% of the total HSC pool (Woll et al., 2014). A paradox inherent in MDS is that although the MDS-propagating clone has increased competitive ability, its expansion ultimately results in bone marrow failure. The presence of MDS stem cells and dying progenitor cells confers decreased function to the coexisting normal stem cells, suggesting that MDS stem and progenitor cells create a bone marrow environment that is killing normal hematopoietic stem cells. In our mouse model with unrestrained hematopoietic necrosis, mice die of bone marrow failure with the majority of the features of human MDS. Furthermore, bone marrow from these mice displayed increased competitive repopulating ability against wild type bone marrow. Transplanted mice die of bone marrow failure at four months despite the persistence of wild type bone marrow, suggesting that these necrotic HSC and progenitor cells can kill wild type HSCs to cause bone marrow failure. Our mice thus shed light on how an MDS clone can cause bone marrow failure:

Our overarching hypothesis is that programmed necrosis in MDS cells triggers an inflammatory response that kills normal hematopoietic stem cells. This in term enables mutant stem and progenitor cells to expand and take over the bone marrow, thus driving bone marrow failure. Interrupting the cell death signaling pathway or altering the inflammatory signaling pathway has

the potential to prevent cell death and re-establish bone marrow homeostasis for therapeutic benefit.

Study design: we propose to use mouse models of both genetic mutations found in MDS and unrestrained necroptosis to characterize the death and inflammatory pathways, cytokines elicited by necrotic cell death, and dynamics of cell proliferation in HSC and progenitor populations using multiplex cytokine arrays and intracellular flow cytometry. We will then inhibit key pathways to determine the impact on hematopoietic homeostasis, and determine whether we can prevent bone marrow failure.

Impact: The goal is to identify how HSCs and progenitor cells harboring MDS mutations execute cell death, and how they kill normal HSCs, and determine whether interrupting this cell death can rescue bone marrow function. As most MDS patients die of cytopenias, this therapy, if successful, will directly address the major morbidity of this disease through targeted therapeutic treatments. We acknowledge that one potential side effect of our strategy, may be accelerated transformation to leukemia, thus we will initially test our hypotheses in mouse models. However, we postulate that expansion of the MDS clone potentiates the acquisition of additional mutations that allow progression to AML, and that intervening in the signaling that expands this clone may actually increase the latency to transformation.

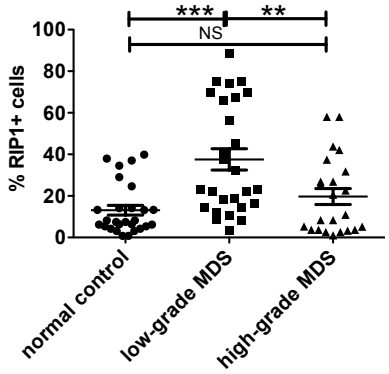

Figure 1: *Human MDS patient bone marrow samples display increased RipK1 staining relative to normal control bone marrow.* FFPE sections from 37 de novo MDS cases and 36 normal control cases were stained with antibodies to Ripk1 and pMLKL as necroptosis markers and cleaved Caspase 3 as an apoptosis marker. The MDS specimens included 20 cases of lower-grade MDS and 17 cases of higher-grade MDS. All cases were reviewed and the diagnosis confirmed by board-certified hematopathologist. Whole slide images were obtained using Aperio Versa 200 (Leica Microsystems). We developed a pipeline for statistical analysis to evaluate the immunofluorescence of stained and scanned images using CellProfiler (Broad Institute) image analysis software. For each data point, (slide) over 10,000 cells were analyzed.

2. Keywords

Programmed cell death, hematopoiesis, hematopoietic stem cell, inflammation, bone marrow failure, Myelodysplastic syndrome, necroptosis, apoptosis, electron microscopy, RipK1 kinase

3. Accomplishments

The accomplishments for the first year of this grant are outlined below and in the attached manuscript that has been submitted to *Blood.* We have revised the manuscript to address all reviewer concerns and are ready to resubmit it for consideration for publication.

Goals:

1. Obtain regulatory approval- completed
2. Define cell death signaling of HSC and progenitor cells
3. Determine how cells die in competitive repopulation
4. Determine cytokine profile of bone marrow
5. Determine the effects of pharmacologic inhibition
6. Determine the effect of inhibition of inflammation on competitive repopulation
7. Determine the effects of inhibition of inflammation on cytokine production

Major activities:

The major activity has been characterization of the mouse models outlined in the original proposal

Specific objectives:

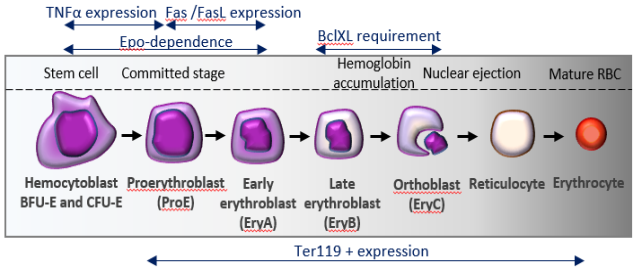

Figure 2: Schematic of erythropoiesis. Erythropoietin responsive early erythroid progenitor cell numbers are responsive to TNFα and Fas death receptor signaling

Aim 1: Define cell signaling of HSC and progenitor cells in mouse models of unrestrained cell death

Characterize cell death in mouse models of unrestrained necrosis

We have created a mouse model in which we have activated necrosis signaling in the hematopoietic system. Deletion of the pro-apoptotic Bcl-2 family members, *Bax* and *Bak* inhibits bone marrow programmed cell death (PCD). Further deletion of the BH3-only member Bid, to generate *BaxBakBid* TKO mice, leads to unrestrained bone marrow necroptosis.

During the initial funding period of the CDMRP award, we have characterized the cell death signaling and cytokine production in hematopoietic stem and progenitor cells in normal and *BaxBakBid TKO* (TKO) mice. (Aim 1, task 1-4) We find that the hematopoietic stem cells in TKO mice are expanded, and display increased proliferation, consistent with resistance to cell death despite increased necroptosis signaling. In contrast, hematopoietic progenitor cells display dramatically reduced cell numbers and reduced proliferation, consistent with increased cell death (attached manuscript, figures 4A-D). Immunofluorescence of bone marrow from TKO mice displays increased Rip1 staining, but no increased activated Caspase 3 staining, consistent with increased programmed necroptosis and inconsistent with apoptosis (attached manuscript, figures 2C-D). Of note, in a parallel study in human MDS patient samples (funded from an independent agency (VA MERIT award), and not funded by this DOD award), we have demonstrated increased RipK1 and not increased cleaved Caspase 3 staining. Transmission Electron Microscopy of bone marrow as well as myeloid progenitor cells from TKO mice displays increased necrosis morphology (attached manuscript, (Figures 2A and 3B, respectively). We then performed primary and secondary competitive repopulation experiments of wild type and TKO bone marrow. Consistent with protection of HSCs from cell death, TKO mice display increased competitive repopulating ability in primary transplants (attached manuscript, Figures 5 A, E). However, transplanted TKO mice develop progressive cytopenias and bone marrow failure (attached manuscript, Figures 5B-D). Necropsy of transplanted mice reveals increased TNF alpha production in multiple organs, most prominent in the lungs (Attached manuscript, Figure 6A). Consistent with the progressive cytopenias observed in primary transplants, TKO bone marrow fails to repopulate in secondary transplantation experiments (attached manuscript, Figure 5G). These results are consistent with a model in which increased necroptotic cell death in progenitor cells results in an inflammatory microenvironment. The initial response of the bone marrow is increased proliferation of HSCs. Ultimately, the increased proliferation and inflammatory microenvironment result in Hematopoietic stem cell exhaustion with failure to reconstitute in secondary transplantation.

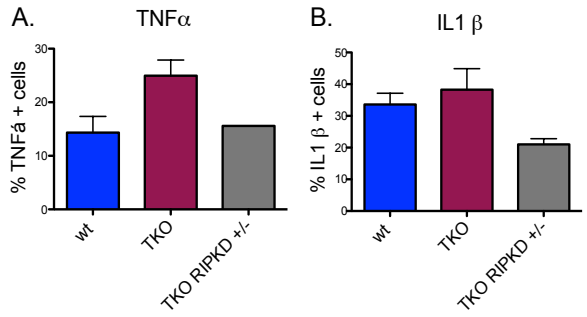

Figure3: Genetic loss of RipK1 function restores normal RBC progenitor numbers and ameliorates extramedullary hematopoiesis of TKO and *Tet2-/-* bone marrow (A, B) and spleens (C,D).

As programmed necrosis results in release of DAMPS that create an inflammatory microenvironment, we hypothesized that our TKO bone marrow would display increased cytokine production. We therefore proceeded to evaluate cytokine production in wild type and TKO LSK and progenitor cells by intracellular cytokine staining. We find that TKO LSK but not progenitor cells display increased TNF alpha and IL-6 production. Both TKO LSK and Progenitor cells display increased IL1-beta production (Attached manuscript, figure 6B-D).

Aim 2: Determine whether inhibiting inflammatory signaling or necrosis signaling can reset hematopoiesis and prevent bone marrow failure

We have demonstrated that our mouse model in which we activated necrosis signaling in the bone marrow develops bone marrow failure that results from altered hematopoietic homeostasis with increased progenitor cell death, resulting in HSC exhaustion. We further demonstrate increased cytokine production in the bone marrow of these mice. We hypothesized that normal bone marrow homeostasis and normal hematopoiesis may be restored by either inhibiting programmed necrosis, or by inhibiting the inflammatory response.

Bone marrow failure disorders such as MDS as well as aplastic anemia display increased TNF alpha production. To inhibit the excess TNFα observed in our mice, we treated TKO mice with Enbrel. We are able to partially restore myeloid progenitor cell numbers and proliferation in our TKO mice and partially restore peripheral blood. To inhibit programmed necrosis, we undertook to approaches: pharmacologic inhibition with an inhibitor of RipK1 kinase (7N-1), and genetic inhibition by crossing our mice with a mouse expressing a kinase inactive form of RIPK1.

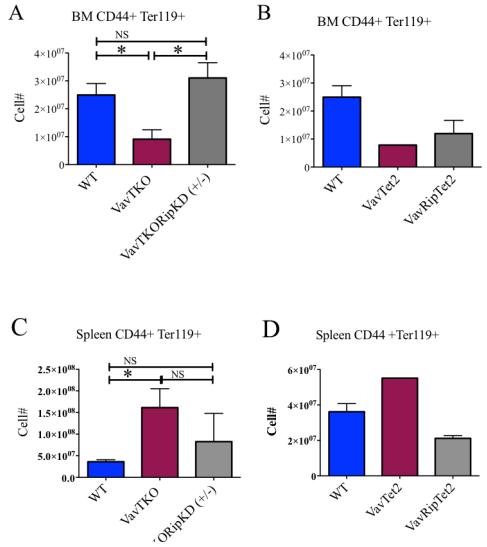

Figure 4: Genetic loss of RipK1 function restores normal bone marrow cytokine production

We found that the pharmacologic inhibition of RipK1 was complicated by toxicity. Despite careful titration of 7N-1, we were not able to arrive at a therapeutic dose (data not shown but available upon request). Genetic inhibition of RipK1 required a complicated cross (5 alleles) and was time-intensive. However, we were able to demonstrate a complete rescue of normal peripheral blood counts in TKO mice with 1 allele of kinase inactive RipK1 (Attached manuscript, Figure 4F-H). Furthermore, we were able to partially rescue normal myeloid progenitor cell numbers (Attached manuscript, Figure 6D-E). As we completely rescued peripheral blood counts, with only partial rescue of progenitor cell numbers. As erythropoiesis is known to be regulated by programmed cell death (Figure 2) we further investigated erythrocyte precursors. We were able to fully rescue erythroid progenitor cell numbers. TKO mice develop extramedullary hematopoiesis with a markedly increased spleen size. We were able to prevent the development of splenomegaly and extramedullary hematopoiesis in TKO mice with one allele of kinase inactive RipK1. We have hypothesized that the increased inflammatory cytokines noted in TKO mice were a result of necroptotic cell death. We therefore predict that inhibition of necroptosis will prevent cytokine production. We are able to prevent TNF alpha and IL-1 beta production in TKO bone marrow with one allele of kinase in active RipK1 (Figure 4).

Our TKO mice with increased necroptosis closely resemble human MDS, and we have demonstrated increased necroptosis signaling in human MDS patient samples.

Next generation sequencing efforts have identified recurrent mutations in MDS, including Asxl1 and Tet2. Despite the identification of these mutations, the mechanism whereby these

mutations result in bone marrow failure has not been identified. We have obtained mice harboring these mutations and crossed them with kinase inactive RipK1. These experiments are ongoing as the crosses have just been completed. We are able to completely restore normal erythroid progenitor cell numbers and prevent extramedullary hematopoiesis and splenomegaly in Tet2 KO mice with one allele of kinase inactive RipK1 (Figure 3A-D). Preliminary evidence suggests that we can also partially restore normal hematopoiesis in ASXL1 mice (data not shown). Ongoing experiments are focused on completing the evaluation of the impact of inactivating necroptosis on HSC and progenitors in these mouse models of MDS. To our knowledge, this is the first demonstration of rescue of hematopoiesis by a defined genetic pathway in a genetic model of MDS.
The experiments investigating the impact of necroptosis on hematopoiesis in our TKO mice was submitted and a revised manuscript addressing all reviewer concerns is ready for resubmission to *Blood* (manuscript attached).

Opportunities for training and professional development

The above work has provided the opportunity for training in hematopoiesis for a graduate student and research associate in my laboratory.

Dissemination to communities of interest

I had the opportunity to present this work at the Edward P Evans Foundation symposium in 2016, comprised of investigators involved in investigation related to MDS.
In addition, I have presented this work at the Gordon Conference for Programmed cell death in 2016. I was an invited speaker and presented this work at the VA-sponsored symposium on inflammation and cancer in honor of Dr. Ann Richmond this fall at Vanderbilt University.

Plan to accomplish goals

I plan to continue the analysis of mouse models of MDS mutations. We have bred all of our models to the Rip1kinase inactive mouse and now have sufficient numbers to complete the analysis. We will follow up on our re-submission of a revised version of our first manuscript, and plan to write up an additional manuscript on the role of necroptosis in erythropoiesis in our mouse model of unrestrained necroptosis as well as our mouse models harboring MDS mutations. We are also leveraging these preliminary results to apply for additional funding to continue these promising studies.

4. Summary and impact:

Hematopoiesis is a dynamic system that requires careful balance between cell division, differentiation, and cell death. The two major modes of programmed cell death (PCD), apoptosis and recently recognized necroptosis, share molecular machinery, but diverge in outcome with important implications for the microenvironment: apoptotic cells implode and are removed in an immune silent process, whereas necroptotic cells leak cellular contents that incite inflammation. Given the importance of cytokine directed cues for hematopoietic cell survival and differentiation, the potential impact of biasing cell death fate to necroptosis on hematopoietic homeostasis is substantial and poorly understood. Here we present a mouse model with increased bone marrow necroptosis: deletion of the pro-apoptotic Bcl-2 family members, Bax and Bak inhibits bone marrow PCD, further deletion of the BH3-only member Bid, to generate BaxBakBid TKO mice, leads to unrestrained bone marrow necroptosis. TKO mice display loss of progenitor cells leading to increased stem cell proliferation and stem cell exhaustion, and increased cytokine production, culminating in bone marrow failure (BMF). Importantly, genetic

inactivation of Rip1 kinase restores normal cytokine production, normal hematopoiesis, and normal erythropoiesis in TKO mice as well as mice harboring deletion of the MDS-associated gene *Tet2.* To our knowledge, this is the first demonstration of rescue of hematopoiesis by a defined genetic pathway in a mouse model of MDS. TKO bone marrow is hyper cellular with abnormal differentiation, resembling the human disorder Myelodysplastic syndrome (MDS), and we demonstrate increased necroptosis in MDS bone marrow. Finally, we show that Bid impacts necroptotic signaling through modulation of Caspase-8-mediated Rip1 degradation. We thus demonstrate that dysregulated necroptosis in hematopoiesis promotes bone marrow progenitor cell death that incites inflammation, impairs hematopoietic stem cells, and recapitulates the salient features of the bone marrow failure disorder, MDS.
We have accomplished the first step on the way to our goal to provide proof of concept that necrosis and/or inflammatory signaling can be targeted for therapeutic benefit in bone marrow failure syndromes such as MDS with the long-term goal of improving patients cytopenias and preventing disease progression to benefit MDS patients.

5. Changes/problems

As noted above, despite a careful titration, we were unable to rescue hematopoiesis using the Rip1 kinase inhibitor 7N-1. We have therefore proceeded to cross our mice to the Rip1 kinase inactive knock in mice developed by Michelle Kelliher as well as GSK with promising results. We would note that the cross involved 5 alleles to inactivate Rip1 kinase in our mouse model of increased necroptosis, resulting in some significant delay and additional expenditures on mouse costs. We do feel that this has been a highly productive cross that has given significant insight into bone marrow failure regulated by necroptosis as well as mouse models of MDS.
In addition, we uncovered an unanticipated finding in erythropoiesis that is highly relevant to MDS as a number of the major morbidities of this disease are due to anemia. Furthermore, understanding the cell death pathways that drive anemia will be highly relevant to a number of other disorders that are prevalent in the Veteran population.

Changes to the use or care of animals, biohazards

None

Changes to care of human subjects

N/A

6. Products

Journal publications:
Our revised publication to be submitted to *Blood* is attached.

Presentations

2016	Invited Speaker: Gordon Research Conference. New concepts in Cell Death research: From Basic Mechanisms to Clinical Opportunities (Girona, Spain)
2016	Invited speaker, Edward P Evans Foundation Symposium Necrosis regulation of bone marrow function (Nashville, TN)
2017	Invited speaker: Center for Mitochondrial and Epigenomic Medicine

Full length Bid maintains mitochondrial cristae structure and function – Integrating a gene-based PheWAS (PrediXcan) approach and mouse models to probe roles in human disease
(Philadelphia, PA)

2017 Invited Speaker: Third National Veterans Health Affairs Research Conference: Cancer Immunotherapy: Advances and Challenges
Necrosis regulation of bone marrow function
(Nashville, TN)

Technology or Techniques

None

Inventions, patent applications, and/or licenses

None

Other products

None

7. Participants and other collaborating organizations

NAME	SANDRA ZINKEL	QIONG SHI	YULIYA HASSAN
PROJECT ROLE	**PI**	**Senior research scientist**	**Research Assistant II**
RESEARCH IDENTIFIER			
NEAREST PERSON MONTHS WORKED	**1.8**	**4**	**2.4**
CONTRIBUTION TO PROJECT	**Dr. Zinkel designs experiments, oversees execution of experiments, analyzes data and writes papers**	**Qiong Shi executes experiments with minimal supervision. She participated in the analysis of TKO mice**	**Yuliya Hassan maintains the mouse colony, manages animal husbandry, genotypes animals and performs analysis of mouse models**
FUNDING SUPPORT	**DOD, VA MERIT**	**DOD, VA MERIT**	**DOD, VA MERIT**

There has been no change in active support of the above individuals since the original reporting period

Other organizations involved as partners

None

8.Special reporting requirements:

None

9.Appendices

Revised Manuscript to be submitted to *Blood.*

Loss of Bid-regulated necrosis inhibition leads to myelodysplasia and bone marrow failure in mice

Running Title: Bid restrains necroptosis to preserve HSPC function

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Scientific Category

Hematopoiesis and stem cells

Key Points:

A new role for a recognized pro-apoptotic protein in programmed cell death fate links hematopoietic necroptosis to bone marrow failure.

Unrestrained bone marrow necroptosis promotes inflammatory cytokine production that impairs HSPC function.

Abstract

Hematopoiesis is a dynamic system that requires careful balance between cell division, differentiation, and cell death. The two major modes of programmed cell death (PCD), apoptosis and recently recognized necroptosis, share molecular machinery, but diverge in outcome with important implications for the microenvironment: apoptotic cells implode and are removed in an immune silent process, whereas necroptotic cells leak cellular contents that incite inflammation. Given the importance of cytokine directed cues for hematopoietic cell survival and differentiation, the potential impact of biasing cell death fate to necroptosis on hematopoietic homeostasis is substantial and poorly understood. Here we present a mouse model with increased bone marrow necroptosis: deletion of the pro-apoptotic Bcl-2 family members, Bax and Bak inhibits bone marrow PCD, further deletion of the BH3-only member Bid, to generate BaxBakBid TKO mice, leads to unrestrained bone marrow necroptosis. TKO mice display loss of progenitor cells leading to increased stem cell proliferation and stem cell exhaustion, and increased cytokine production, culminating in bone marrow failure (BMF). Genetic inactivation of Rip1 kinase restores normal cytokine production as well as normal hematopoiesis. TKO bone marrow is hyper cellular with abnormal differentiation, resembling the human disorder Myelodysplastic syndrome (MDS), and we demonstrate increased necroptosis in MDS bone marrow. Finally, we show that Bid impacts necroptotic signaling through modulation of Caspase-8-mediated Rip1 degradation. We thus demonstrate that dysregulated necroptosis in hematopoiesis promotes bone marrow progenitor cell death that incites inflammation, impairs

hematopoietic stem cells, and recapitulates the salient features of the bone marrow failure disorder, MDS.

Introduction

Programmed cell death (PCD) is required to maintain homeostasis in dynamic systems such as hematopoiesis in adult organisms [1]. The two main forms of PCD, apoptosis and necroptosis, result in markedly different outcomes with important implications for the cellular microenvironment: apoptotic cells are removed in an immune silent process, while necroptotic cells incite inflammation [2]. In hematopoiesis, cells respond to chemokine and cytokine-directed micro environmental cues to maintain homeostasis [3]. (Inflammation and hematopoiesis- stem cell function)

Programmed necrosis or “Necroptosis” occurs in response to TNF, Fas, TRAIL as well as certain TLR ligands. Necroptosis, by virtue of the early rupture of the cellular plasma membrane, can promote inflammation through release of endogenous molecules such as DNA or membrane lipids known as damage-associated molecular patterns (DAMPs). Because certain of the cytokines released such as TNFα can also serve as ligands, the process of necroptosis has the potential to amplify inflammation within the microenvironment. TNFα is produced by cells of the myeloid lineage in response to DAMPs. Within the bone marrow microenvironment, TNFα can impair hematopoietic stem cell colony-forming ability as well as the ability to sustain multilineage differentiation. The potential impact of biasing cell death fate to necroptosis on hematopoietic homeostasis is therefore substantial and poorly understood.

The upstream molecular signaling machinery through death receptors such as TNFα (Tumor necrosis factor receptor alpha), FAS, and DR4/5 (Death receptor 4/5) is shared among apoptosis and necroptosis, but diverges prior to activation of effector Caspases or Rip kinases culminating in apoptotic or necroptotic death, respectively. Seminal studies of genetic mouse

models further demonstrate that the upstream activators of apoptosis, FADD and Caspase-8, act as key inhibitors of necroptotic cell death during embryonic development [4–10]. This strongly suggests that the molecular interactions that commit a cell to necroptosis through Rip1 kinase activity or to apoptosis through Caspase-8 activity lie downstream of receptor-ligand interactions, yet must take place before the preferential activation of either effector Caspases or Rip kinases.

The Bcl-2 (B-cell lymphoma) family of proteins are situated at this central decision point of cell death fate, functioning downstream of death receptor signaling yet before activation of executioner Caspases and cell death. While their central role in apoptotic cell death is well understood, the mechanistic link between the Bcl-2 family and necroptotic cell death has not been described. In particular, the BH3-only family member Bid, acts as a sensor and amplifier of death receptor signaling. Upon activation of death receptors (TNFα, FAS) Bid is cleaved by Caspase-8 [11,12], and translocates to the mitochondria, where it directly or indirectly activates Bax/Bak(Walensky paper). Activated Bax and Bak hetero oligomerize to form a pore and initiate mitochondrial outer membrane permeabilization (MOMP), resulting in activation of the apoptosome and execution of apoptotic cell death. The interaction of Bid with Caspase 8 as well as Bid's role in early activation of apoptosis, linking the intrinsic and extrinsic apoptotic pathways is therefore well-established.

Bid has also been shown to function in a pro-survival role in which it acts to restrain cell death execution in certain contexts [13–16]. We propose that this pro-survival function of Bid extends to its role in actively restraining necroptosis, mediated through modulation of Caspase-8 activity. To test whether Bid may influence cell death fate, we generated two mouse models. First, we conditionally deleted Bax using Vav-Cre, and crossed this to a mouse model with germ line deletion of Bak in order to create hematopoietic-specific BaxBak double knockout (DKO) mice. This cross results in the loss of intrinsic apoptotic execution in hematopoiesis, however importantly leaves the upstream signaling pathway of interest intact. To specifically assess the

role of Bid, we crossed these mice with Bid-/- mice to create VavBaxBakBid triple knockout (TKO) mice [17,18]. These models allow us to determine BaxBak independent roles of Bid at the central decision node upstream of the mitochondria yet still downstream of the receptors and immediate DISC complexes [19,20].

We confirm that deletion of BaxBak completely blocks apoptotic cell death but is not sufficient to initiate necroptotic cell death. Further deletion of a third pro-apoptotic protein, Bid, however, leads to robust activation of necroptosis and specifically early death due to bone marrow failure (BMF). Furthermore, we demonstrate that the presence of Bid correlates with Rip1 degradation and apoptosis execution through an Z-IETD FMK-inhibitable activity mediated by Caspase 8 in myeloid progenitor cells. We have thus created genetic mouse models in which hematopoietic cells are protected from PCD (VavBaxBak DKO), and show that by removing Bid, a third pro-apoptotic Bcl-2 family member (VavBaxBakBid TKO), we switch cell death fate to necroptosis from apoptosis (wild type) under homeostatic conditions in mouse bone marrow.

In competitive reconstitution studies, mice transplanted with TKO as well as mice transplanted with DKO test bone marrow initially display increased competitive reconstitution compared to the wild type control, but develop progressive anemia and thrombocytopenia leading to early mortality, suggesting that the presence of necroptotic hematopoietic cells exerts a cell-extrinsic effect on hematopoietic function of normal cells. TKO but not DKO bone marrow fails to reconstitute in a secondary transplant, demonstrating that increased necroptosis impacts hematopoietic stem cell repopulating ability.

Importantly, genetic inactivation of Rip1 kinase by crossing TKO mice to Vav-RipK1 KI mice (K45A or D138N) fully restores peripheral blood counts and substantially increases progenitor cells percentages and proliferation as measured by BrDU incorporation. In addition, consistent with a role of necrosis-driven cytokine production, genetic inactivation of Rip1 kinase in TKO mice decreases cytokine production. Furthermore, treatment of TKO mice with the decoy TNFR, Enbrel, partially restores progenitor cells, TKO anemia and thrombocytopenia, further

demonstrating that the preferential progenitor cell death and resultant cytopenias observed are driven by necroptosis and amplified, at least in part, by increased inflammatory cytokine production. Notably, we also demonstrate increased necroptotic signaling and increased necrotic morphology by electron microscopy in the human bone marrow failure disorder, Myelodysplastic Syndrome (MDS), demonstrating the impact of necroptosis signaling on normal bone marrow function and its relevance to disease.

Methods

Mice

Details for the mouse strains used in this study are described in supplemental Materials, available on the Blood web site.

Flow Cytometry

Cells from peripheral blood and bone marrow were stained and analyzed according to previously described protocols

Immunoblotting and immunofluorescence

Details of immunoblotting and immunofluorescence are described in supplemental materials and methods.

Competitive Reconstitution

Competitive reconstitutions studies were performed according to previously described protocols

RESULTS

Vav BaxBakBid TKO mice die of Bone marrow failure (BMF)

We developed mouse models that enable us to study PCD regulation at the central decision node upstream of the mitochondria yet still downstream of the receptors and immediate DISC complexes (Fig. 1A). [19,20]. First, we conditionally deleted Bax using Vav-Cre, and crossed this to a mouse model with germ line deletion of Bak in order to create hematopoietic-specific BaxBak double knockout (DKO) mice. This cross results in the loss of intrinsic apoptotic execution in hematopoiesis, however importantly leaves the upstream signaling pathway of interest intact. To specifically assess the role of Bid, we crossed these mice with Bid-/- mice to create VavBaxBakBid triple knockout (TKO) mice [17,18]. These models allow us to determine BaxBak independent roles of Bid at the central decision node upstream of the mitochondria yet still downstream of the receptors and immediate DISC complexes (Figure 3I) [19,20].

As shown in Fig. 1B and 1C, Vav-Cre efficiently deletes Bax in the bone marrow and spleen of DKO and TKO mice. Consequently, there is no detectable mRNA by RT-PCR (Fig 1B) or protein by Western blot (Fig 1C). Phenotypes present in Vav-Cre TKO mice distinct from Vav-Cre DKO mice indicate BaxBak- independent functions of Bid, allowing us to interrogate Bid-dependent upstream signaling events driving commitment to apoptosis versus necroptosis in hematopoiesis.

Vav-CreBaxBak DKO (DKO mice) die predominantly (89%) of lymphoid leukemia or myeloproliferative disease (MPD) that can be transferred to recipient mice, consistent with loss of mitochondrial–mediated death (Fig. 1D, E, G) [21,22]. In contrast, mice harboring loss of Bid in addition to Bax/Bak (TKO mice) display decreased survival relative to Bid+/+ (WT), Bid-/- (Bid KO), and BaxBak DKO mice (Fig. 1D). 66% of TKO mice die of bone marrow failure (BMF), with decreased hemoglobin concentrations (red blood cell (RBC) numbers) and platelet numbers (Fig. 1E, F). In addition, TKO mice display extensive myeloid dysplasia (abnormal differentiation and development) with age: I) neutrophils (hyper-segmentation), II) megakaryocytes (hypo-lobulation), and III) erythroid precursors (binucleation and intrachromosomal bridging)(Fig.1H) [23]. Transformation to leukemia/MPD only occurs in 22% of

TKO mice. This marked difference in hematopoietic phenotype with additional Bid deletion establishes that Bid can regulate hematopoietic homeostasis independent of its Bax/Bak activator role.

TKO bone marrow dies by necroptosis

The presence of markedly decreased cells in TKO blood despite preserved bone marrow cellularity (Fig. S1B) suggests increased cell death in the bone marrow. We performed transmission electron microscopy (TEM) to examine cellular morphology, a defining feature of both apoptosis and necroptosis. Image comparison of Bid+/+ and TKO cells reveal apoptotic morphologies characteristic of apoptosis (e.g. pyknotic nuclei, cell membrane and organelle shrinkage) and necroptosis (e.g. membrane integrity loss, cell membrane and organelle swelling), respectively (Fig. 2A). Image quantification reveals necroptotic morphology in 25% (TKO), 7% (Bid+/+) and 10% (DKO) of bone marrow cells (Fig. 2B). Necroptotic PCD signaling is executed by Rip1 and Rip3 [24]. Accordingly, Rip1 levels are increased by immunofluorescence in TKO but not Bid+/+, Bid-/-, or DKO bone marrow (Fig. 2C). No significant cleaved Caspase-3 was observed in DKO or TKO bone marrow (Fig. 2D and 2E), inconsistent with apoptosis as the primary cell death mechanism in this setting. Immunofluorescence data further supports necroptotic PCD in TKO bone marrow, establishing that while loss of BaxBak prevents MOMP and apoptosis in the bone marrow, the additional loss of Bid induces necroptotic cell death.

TKO myeloid progenitors die by necrosis

We next generated Hox11 immortalized myeloid progenitor cells (MPCs), from the bone marrow of Bid +/+, Bid -/-, MxBaxBak DKO, and MxBaxBakBid TKO mice [13] ,and treated these MPCs with TNFα plus Actinomycin D (TNFα/ActD) to activate TNFR signaling and block survival signaling. As expected for Type II cells, Bid-/- and DKO cells exhibited less death (Annexin V^+/PI^+) in response to TNFα/ActD [25]. Notably, DKO MPCs do not undergo necroptosis following TNFα/ActD, suggesting that inhibiting apoptosis is insufficient to elicit necroptotic cell death.

TKO and Bid+/+ cells display similar death kinetics (Fig. 3A, I), and Bid+/+ but not TKO MPCs displayed increased cleaved Caspase-3 (Fig. 3A, II). Additionally, following TNFα/ActD, Annexin V^+ TKO MPCs exhibit increased size (Fig. S2A), consistent with necroptotic cell death. TEM of TNFα/ActD-treated MPCs reveals predominantly apoptotic morphology in Bid+/+ cells, whereas TKO cells display overwhelmingly necrotic cell morphology (Fig. 3B). Untreated MPCs show minimal cell death (Fig. S2B). Our MPCs therefore behave in a manner consistent with death receptor signaling: Bid+/+ (wild type) MPCs undergo apoptosis in response to TNFα/ActD; removal of Bax and Bak prevents cell death. Importantly, removal of Bid in addition to Bax and Bak results in necroptotic cell death in response to TNFα/ActD, uncovering a novel role for Bid to inhibit necrotosis.

TKO MPCs display increased necroptotic signaling

Phosphorylation of Rip1 has been shown to stabilize its association with a pro-necroptotic complex, and activate necroptotic kinase activity [26]. TKO but not Bid+/+, Bid-/-, or DKO MPCs displayed constitutive and increased kinetics of Rip1 phosphorylation manifested by a phosphatase-sensitive shifted band [27] (Fig. 3C and Fig. S2C and S2D). Interestingly, Bid levels were inversely correlated with Rip1 phosphorylation(Fig. S2E). In addition, TKO but not Bid+/+ or DKO MPCs display increased MLKL trimerization with or without LPS stimulation, further supporting constitutive necroptosis signal execution in TKO MPCs (Fig. 3D) [28]. These results suggest that loss of Bid in addition to Bax and Bak is sufficient to stimulate necroptotic signal execution, consistent with release of a Bid-directed brake on necroptosis

Bid regulates Rip1 stability via modulating Caspase-8 activity

The above studies reveal that the levels of Rip1 vary markedly between genotypes (Fig. 3C). In particular we note that Rip1 levels in DKO MPCs, which express increased levels of Bid (Fig. S2E), are decreased relative to TKO MPCs (Fig 3C). Importantly, reintroduction of Bid into TKO MPCs by retroviral transduction results in decreased Rip1 levels (Fig 3F), demonstrating that the decreased Rip1 observed in DKO MPCs is due to the presence of Bid.

Rip1 can be ubiquitylated or cleaved by proteases such as Caspase-8 [29], and Cathepsins [30] to promote its degradation. Treatment with MG132 (proteasome inhibitor) or Z-VAD-FMK (pan-caspase inhibitor) did not recover full length Rip1 in Bid+/+, DKO, or TKO MPCs (Fig. S3F). Treatment with Z-IETD-FMK (Caspase-8/Granzyme inhibitor) following LPS or TNFα stimulation completely recovered full length Rip1 in DKO MPCs and decreased truncated Rip1 in Bid+/+ and TKO MPCs, indicating that an Z-IETD-FMK-inhibitable enzyme cleaves Rip1 (Fig. 3G). Similar recovery of full-length levels of another Caspase-8 substrate, Cylindromatosis (CYLD), was also observed (Fig. S3G). Furthermore, deletion of Caspase-8 using CRISPR/CAS9 with 2 independent gRNAs, results in an increase in Rip1 levels in Bid +/+ MPCs (Fig. 3H) that is proportional to the degree of Caspase-8 knockdown achieved. While the above results are consistent with a role for Caspase 8 in mediating Rip 1 levels, the inability to restore RIP1 levels with ZVAD suggests either that Caspase 8 exists in a ZVAD-resistant conformation or that another enzyme may also cleave Rip 1 in this setting.

Unrestrained bone marrow necroptosis disrupts hematopoietic homeostasis

Necroptotic cells create an inflammatory microenvironment [31]. Inflammatory cytokines have been shown to impair HSC function [32], suggesting the possibility that bone marrow necroptosis may impair HSC function and hematopoietic homeostasis.

Immunophenotype of hematopoietic stem and progenitor cells (HSPCs) reveals that LSK (Lin^- $Sca1^+$c-Kit^+) cell populations were expanded in TKO but not Bid+/+, Bid -/-, or DKO mice (Fig. 4A). The signaling lymphocyte activating molecule-HSC (SLAM-HSC, LSK $Flt3^{Lo}CD48^-$ $CD150^+$) population [33], more highly enriched for HSCs, continues to expand in TKO but not DKO mice with age. Accordingly, TKO but not DKO SLAM-HSCs displayed increased BrDU incorporation, consistent with an appropriate response to bone marrow stress (Fig. 4B). LT-HSC (Lin^- $Sca1^+$c-Kit^+CD135^{Lo}) populations were not significantly changed between genotypes (Fig. S3A). Importantly, DKO mice do not display increased SLAM-HSC proliferation despite

decreased progenitor cell proliferation (Fig. 4B and 4D), suggesting a distinct defect in hematopoietic homeostasis in DKO vs. TKO mice.

Given the apparent bone marrow stress noted in TKO SLAM-HSCs, we anticipated that progenitor populations would be similarly expanded with increased BrDU incorporation. In contrast, we found that both TKO and DKO myeloid progenitor (Lin^- $Sca1^-$c-Kit^+) populations are decreased (Fig. 4C) and display significantly decreased BrDU incorporation as compared to Bid+/+ mice (Fig. 4D). The expanded SLAM-HSCs with decreased progenitors in TKO mice are consistent with increased sensitivity of the progenitor population to cell death with compensatory HSC proliferation.

Consistent with the increased programmed cell death noted in myeloid cells, TKO but not Bid+/+, Bid-/-, or DKO mice display splenomegaly with increased $Ter119^+$ cells (erythroid), indicative of extramedullary hematopoiesis that is progressive with age (Fig. S3B and S3C). Notably, B cell and monocyte populations are not different between genotypes at necropsy, but TKO T cell populations are expanded (Fig. S3D).

To definitively establish whether the altered hematopoiesis observed in TKO mice was due to unrestrained necroptosis, we crossed our TKO mice to mice in which a kinase inactive mutant of Rip1 (K45A or D138N) is conditionally inserted into the Rip1 locus. Expression of one copy of Rip1 KD is sufficient to restore normal hematopoiesis as measured by peripheral blood counts (Red blood cells, hemoglobin, and hematocrit, Fig. 4F-H). In addition, bone marrow cell numbers, myeloid progenitor cell numbers and proliferation are partially restored (Fig. 4I-K). We thus establish that increased necroptosis drives the altered hematopoiesis observed in TKO mice.

TKO cells outcompete Bid+/+ cells, but fail to maintain hematopoiesis in competitive repopulation experiments

To stringently evaluate TKO HSPC function, we required test (TKO) bone marrow to compete with normal bone marrow to repopulate a lethally irradiated congenic mouse (competitive repopulation). Accordingly, we injected a 1:1 ratio of TKO (Ly45.2+) to wild type (Bid +/+) (Ly45.1+) bone marrow into lethally irradiated wild type (Bid +/+) (Ly5.1+) mice, and evaluated peripheral blood for Ly45.2+ and Ly45.1+ mononuclear cells. Two additional cohorts of mice were examined in which a 1:1 ratio of wild type (Ly5.2+) to wild type (Ly5.1+) or a 1:1 ratio of DKO (Ly5.2+) to wild type (Ly5.1+) marrow was transplanted into lethally irradiated wild type (Ly5.1+) mice. Surprisingly, both DKO and TKO bone marrow displayed increased repopulating ability relative to Bid+/+ marrow (Fig. 5A). However, peripheral blood counts reflected bone marrow stress that was more severe in TKO mice, with decreasing RBC counts (anemia) and platelets (thrombocytopenia) and increasing platelet size (Mean platelet volume) (Fig. 5B, 5C, and 5D).

Hematopoietic stem and progenitor compartments reflect a distinct phenotype of DKO and TKO HSPCs

To further explore how altered cell death mechanism impacts non-cell autonomous interactions in the HSPC and progenitor compartment, we evaluated progenitor, LSK, and SLAM HSC populations post 20 weeks in primary competitive repopulation experiments. Similar to un-transplanted mice, DKO and TKO-transplanted progenitor cell numbers were similar, and markedly decreased relative to Bid +/+ (wild type) transplanted mice. Interestingly, wild type progenitors in TKO transplanted mice were decreased relative to wild type progenitors in DKO transplanted mice, suggesting the possibility that the presence of dying TKO progenitors may impair co-transplanted wild type progenitors. Strikingly, whereas DKO and wild type LSK cell numbers are similar, TKO bone marrow displays a substantial increase in LSK (~7x) and SLAM HSC (~2x) numbers, and a relative decrease in progenitor cells (Fig. 5E). In contrast, DKO HSPCs are ~2 fold decreased relative to wild type HSPCs, consistent with bone marrow

crowding due to increased mature cells that did not die. Beginning at 20 weeks, mice transplanted with both DKO and TKO bone marrow but not Bid+/+ bone marrow alone, began to die. In addition, TKO -transplanted mice displayed evidence of BMF (Fig. 5 B-D, S4A and S4B), with decreased RBCs on peripheral blood smear and increased bone marrow debris. Examination of blood and bone marrow from mice transplanted with TKO bone marrow reveals dysplasia and bone marrow cell death similar to that observed in TKO mice. At the time of death, TKO cells represented 80-90% of the bone marrow. Despite the presence of 10-20% wild type bone (Bid +/+) marrow, hematopoiesis was not maintained, suggesting a cell-extrinsic effect of TKO bone marrow on wild type HSPCs.

Secondary transplant reveals decreased TKO HSPC repopulating ability.

To further evaluate HSPC function, and to compare DKO and TKO bone marrow reserve, we performed a secondary transplant. DKO bone marrow continues to out-compete wild type bone marrow even in secondary transplant conditions, indicating continued HSPC self-renewal capacity (Fig 5G). In contrast, TKO bone marrow displays strikingly decreased competitive repopulating ability, consistent with decreased HSPC self-renewal capacity (exhaustion) in secondary transplant conditions (Fig. 5G). We thus demonstrate that increased necroptosis impairs long-term HSPC function, resulting in HSPC exhaustion.

Mice transplanted with TKO bone marrow display increased TNFα production

Notably, mice transplanted with TKO but not wild type (Bid +/+) nor DKO bone marrow displayed marked inflammation in the lungs, kidney, and liver upon necropsy. Immunofluorescence revealed increased TNFα expression in lungs from mice transplanted with TKO but not wild type nor DKO bone marrow (Fig. 6A), suggesting that transplantation of TKO but not wild type or DKO cells promotes inflammatory cytokine production. Accordingly, TKO but not wild type or DKO bone marrow displayed significantly increased TNFα, IL-6 and IL-1 expression, as measured by intracellular flow cytometry, following LPS stimulation (200 ng/mL)

(Fig. 6B-D). The above results are consistent with inflammation induced by dying TKO cells leading to increased TNFα, which kills wild type and TKO HSPCs, producing BMF.

Treatment with TNF decoy receptor (Enbrel) restores HSPCs and improves cytopenias in TKO mice

We next sought to determine whether inhibiting TNFα could improve TKO cytopenias. We treated a cohort of Bid+/+, DKO and TKO mice with Enbrel to inhibit TNFα. Enbrel treatment increased the number of TKO myeloid progenitor cells and $BrDU^{+}$ myeloid progenitor cells such that were not significantly different from the corresponding Bid +/+ cell numbers (Fig. 5E). Enbrel treatment also improved RBC and platelet counts in Enbrel-treated TKO mice (Fig. 5F), consistent with a role of necroptosis-induced TNF in TKO BMF.

The human disease MDS demonstrates increased Rip1 and Phospho-MLKL expression, suggesting increased necroptic signaling

We demonstrated above that increased necroptosis in mouse bone marrow results in BMF with a cellular bone marrow, prominent dysplasia, and a small frequency of transformation to leukemia, phenocopying the human BMF disorder, Myelodysplastic Syndrome (MDS). Increased cell death in MDS bone marrow has been attributed to apoptosis. However, review of the published data in light of current knowledge reveals that early studies measured cell death using techniques that do not distinguish between apoptotic and necroptotic cells [34]: increased in situ end labeling, increased TUNEL staining, or increased DNA laddering on gels [35–38]. Increased Caspase-3 activity was seen in cultured MDS bone marrow [39], but in only 10% of MDS samples when measured directly ex vivo [40]. Thus, evidence to date does not distinguish between apoptotic and necroptotic cell death in MDS bone marrow in vivo.

To determine whether necroptotic cell death may play a role in MDS, we investigated necroptosis and apoptosis in MDS patient bone marrow samples. Immunofluorescence for Rip1 as well as pMLKL revealed increased expression in all samples of RCMD, and 50% of RAEB-1

and -2 subtypes of MDS in our 22-patient cohort (Fig. 7A, C, D) consistent with increased necroptosis signaling in MDS bone marrow. Conversely, staining for cleaved Caspase-3 reveals modest staining in only a few samples, including controls (Fig. 7B), inconsistent with significant apoptosis in our cohort of patients. We also observed an inverse correlation between Rip1 and Bid expression in several MDS but not control patient samples (Fig. S5A). We further obtained bone marrow from three patients with normal bone marrow, early MDS, and RAEB, at the time of biopsy for Transmission Electron Microscopy (TEM) to minimize cell death artifact. Early MDS cells clearly show marked necrosis morphology relative to RAEB or control bone marrow (Fig. 7E), While this study does not rule out a role for apoptosis in a subset of MDS patients, our study clearly implicates necroptosis signaling in MDS.

Discussion

Although the impact of necroptosis signaling on early embryonic development has been carefully dissected, the role of necroptosis in dynamic systems such as hematopoiesis under homeostatic conditions has not been determined. We have developed a novel set of mouse models tuned to undergo apoptosis (wild type) or necroptosis (BaxBakBid TKO), to explore the impact of necroptotic PCD on hematopoiesis. Using these mouse models, we demonstrate that increased necroptotic cell death in the bone marrow, leads to loss of the progenitor cell populations with compensatory expansion and proliferation of SLAM HSCs. We further demonstrate in competitive repopulation experiments that necroptotic cells can cause bone marrow failure, even in the presence of normal hematopoietic cells, suggesting that necroptotic cell death results in a cell extrinsic impairment of both normal and mutant hematopoietic stem cells. We postulate that this cell extrinsic effect is mediated by the release of DAMPS, which promotes the release of inflammatory cytokines such as TNFα (Fig. 6F and 6G) that amplify cell death. Accordingly, treatment of TKO mice with Enbrel partially restores TKO progenitor cells and peripheral cytopenias. Our data demonstrate that, in addition to the degree of cell death,

the mechanism by which cells die can have a dramatic impact on bone marrow homeostasis, and that skewing death to necroptosis results in BMF.

In the absence of Bid in the context of Bax/Bak deletion, Rip1 levels are increased, and necroptosis is enhanced. Multiple lines of evidence have identified Caspase-8 and the Rip kinases as central to the early signaling events that commit a cell to a given cell death fate. Leveraging cell lines from our mouse models, we demonstrate that Bid restrains necroptosis in hematopoiesis through a Bid:Caspase-8 axis, that cleaves and inactivates Rip1 (Fig. 6E). This is consistent with the previous findings [4], in which Rip3 activates an apoptotic cell death pathway in settings where Rip1 is blocked.

Our studies in hematopoietic cells are in agreement with studies focused on BaxBak DKO MEFs and cardiac myocytes in which BaxBak DKO cells are protected from necroptotic stimuli [41]. In addition, we demonstrate a novel role for Bid to restrain necroptosis: loss of Bid function is required for necroptosis execution in the absence of Bax and Bak both in vitro as well as in vivo in hematopoietic cells.

The hematopoietic phenotype of our TKO mice phenocopies the human BMF disorder, MDS, and we demonstrate increased necroptosis signaling in primary MDS bone marrow. Our results thus shed light on how increased programmed necrotic cell death can amplify bone marrow cell death and lead to BMF. Clinical trials targeting TNFα demonstrated therapeutic benefit in pilot studies but not randomized phase 2 trials, suggesting that the excess TNFα noted in MDS can drive cytopenias in this disease, but that targeting TNFα at the receptor level is not sufficient [42,43]. We show in mouse models that genetic introduction of 1 allele of kinase inactive Rip1 is sufficient to restore normal hematopoiesis and ameliorate excess cytokine production. Additional studies will be required to determine whether combining necroptosis and cytokine inhibition can provide additional benefit.

Two recent studies demonstrate BMF due to HSC dysfunction in mice harboring a kinase-inactive form of Rip1 [44], and impaired engraftment of fetal liver hematopoietic cells from Rip1 -/- mice [5] demonstrating that signaling through Rip1 kinase is required for proper HSC function. However, the loss of stem cell function in these mice impeded further evaluation of the impact of Rip1 kinase on hematopoiesis as hematopoietic cell development beyond HSCs is severely attenuated. Our study, designed to interrogate increased necroptosis signaling, demonstrates an expanded HSC population with increased repopulating ability, in agreement with a role for Rip1 kinase signaling to support HSC function. This may occur through Rip1 –mediated activation of NFκB in HSCs. We further show that myeloid progenitors are more sensitive to increased Rip1 signaling and undergo necroptosis that results in bone marrow failure through both cell intrinsic as well as cell extrinsic mechanisms. Our study has important implications for settings in which an insult such as infection or chemotherapy is delivered to the entire bone marrow and induces necroptotic cell death. The ability to intervene to inhibit necroptosis in these settings may provide a mechanism to ameliorate myelosuppression secondary to chemotherapy or viral infections.

Our mouse models provide novel insights into how increased necroptosis impacts hematopoietic homeostasis and HSC function leading to BMF. We further demonstrate that aberrant bone marrow necroptosis contributes to bone marrow failure disorders such as MDS. Substantial data have established the presence of increased cell death and increased inflammation in MDS bone marrow. We now demonstrate increased necroptosis in MDS bone marrow, and elucidate how aberrantly increased bone marrow necroptosis may contribute to the pathogenesis of bone marrow failure disorders such as MDS.

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Author Contributions

PNW, QS, CTSR, SZ and JZ performed experiments. SSZ, PNW and CTSR designed experiments. SSZ and CFL, collaborated on experimental analysis. YF and MS provided patient samples of MDS. All authors analyzed and interpreted data. PNW, SSZ, and CFL wrote the manuscript.

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Figure Legends

Figure 1: Loss of Bax, Bak, and Bid in hematopoietic cells results in development of bone marrow failure.

(A) Schematic of hematopoietic homeostasis and bone marrow failure (BMF) and development of Vav Cre transgenic mouse models to study the role of cell death. (B) Deletion of BAX in bone marrow and spleen determined by RT-PCR (top) and diagram of highlighting Cre Lox recombination with LoxP sites flanking exons 2-4 of BAX gene (bottom). (C) Immunoblot examining expression of Bax, Bak, and Bid, in WT (Bid+/+), VavCreBaxBak (DKO), and VavCreBaxBakBid (TKO) mice. (D) Survival curves of Bid +/+, Bid -/-, DKO, and TKO mice. Statistics demonstrate differences between DKO and TKO animals. Bid+/+ n=4, Bid-/- n=4, DKO n=14, TKO n=22. (E) Cause of death in DKO and TKO mice determined at time of

necropsy. (F) From top to bottom complete blood counts (CBCs) including: RBCs (10^6/μl), hemoglobin (g/dL), and platelet counts (10^6/μl). n=3,4 Bid+/+ and TKO mice, respectively. (G) Bone marrow and cerebrospinal fluid from Bid +/+ mice transplanted with leukemic DKO bone marrow post sub-lethal irradiation. Scale bar denotes 50 microns. (H) Cytospins from the bone marrow of Bid +/+ and TKO mice denoting I) neutrophils, II) megakaryocytes, and III) erythroid precursors. Arrows indicate I) hyper-segmentation, II) hypo-lobulation, and III) binucleation and intra-chromosomal bridging. Scale bars=10 microns. *p< 0.05, ***p< 0.001. Data are representative of mean +/- SEM.

Figure 2: TKO bone marrow dies by necrosis.

(A) Representative transmission electron microscopy (TEM) images from Bid +/+ and TKO mice. Upper image (3,200X magnification), scale bar=2 microns and lower image (15,000X magnification), scale bar= 500 nanometers. (B) Quantitation of apoptotic and necrotic cells from WT, DKO, and TKO TEM. 100 cells with a nucleus from lower magnification images were scored based on cell and organelle morphology. (C) Rip1 fluorescent immunohistochemistry as a marker for necrotic cell death on paraffin-embedded bone marrow sections from Bid +/+, Bid -/-, DKO, and TKO mice. Staining was performed three independent times, scale bar= 50 microns. (D) Fluorescent immunohistochemistry for cleaved caspase-3 as a marker of apoptotic cell death on WT and TKO bone marrow sections as in (C). Fluorescent immunohistochemistry for Bid +/+ liver post-tail vein injection with Fas ligand as a positive control for cleaved caspase-3 staining. **p< 0.01. Data are representative of mean +/- SEM.

Figure 3: Loss of Bid modulates Rip1 signaling in a caspase dependent manner.

(A) Examination of death in myeloid progenitor cells (MPCs). I) MPCs were treated with 25 ng/mL TNFα + 50 ng/mL Actinomycin D (ActD). Viability was determined by Annexin V/ PI staining. II) MPCs treated with TNFα/ActD were stained for cleaved caspase-3 (C. Caspase 3+) by intracellular flow. Experiment was performed three independent times, data are represented as mean +/- SEM. Statistics indicate differences between Bid +/+ versus Bid -/-, DKO, and TKO.

(B) Bid +/+ and TKO MPCs treated with TNFα + Actinomycin D were examined by transmission electron microscopy (TEM). 50 cells with a nucleus were examined and characterized as being apoptotic or necrotic. Arrows indicate apoptotic cells, and asterisks indicate necrotic cells, scale bar=2 microns. Quantitation of cells to the right of images. (C) MPCs unstimulated or stimulated with 250 ng/mL LPS for 10 minutes and 1 hour followed by immunoblot for Rip1. pRip1 indicates Rip1 phosphorylation. Experiment was performed four times. (D) Immunoblot of MLKL trimerization in Bid +/+, DKO, and TKO MPCs following stimulation with LPS. MLKL timer is approximately 150 kDa. Experiment was performed three times. (F) Immunoblot of Rip1 in Bid+/+, DKO, TKO and TKO+ FLAG-Bid MPCs following LPS stimulation. Experiment performed two independent times. (G) Immunoblot of Rip1 in Bid+/+, DKO, and TKO MPCs by immunoblot following stimulation with 250 ng/mL LPS and pre-treatment with 20μM Z-IETD-FMK (inhibitor of Caspase-8). Experiment was performed three independent times. (H) Rip1 levels by immunoblot after knock-down of Caspase-8 utilizing the CRISPR-Cas9 system in Bid +/+ cells. Experiment was performed two times. (I) Diagram of potential role for Bid in the modulation between apoptotic and necrotic cell death. ***p< 0.001, **p<0.01. Data are representative of mean +/- SEM.

Figure 4: TKO mice have decreased myeloid progenitor cells and increased hematopoietic stem cells (HSCs), which can be rescued with a genetic cross to RIP-kinase dead (RIPKD) mice.

(A) Flow cytometry analysis of bone marrow to examine LSK (Lineage$^-$, Sca-1$^+$, c-Kit$^+$) and (B) SLAM-HSC (Signaling lymphocyte activating molecule- hematopoietic stem cell) populations. Mice were examined before onset of sickness. Younger mice were 11-15 weeks old and older mice were 15-20 weeks of age. Bid +/+ n=5, Bid -/- n=5, DKO n=6, TKO Younger n=5, TKO Older n=9 (C) Examination of the number of BrDU+ SLAM-HSCs in bone marrow. Mice were injected with a total of 4mg of BrDU in three doses over 36 hours. Bone marrow was harvested,

depleted for terminal lineages, and stained for flow cytometry. All remaining cells were analyzed by flow cytometry. Mice were 18-20 weeks of age. (D) Numbers of myeloid progenitors (Lineage$^-$, Sca-1$^-$, c-Kit$^+$) as in (A). Mice were 18-20 weeks of age. (E) Number of BrDU+ myeloid progenitors. Numbers of mice for (C), (D), and (E) are as follows: Bid+/+ n=8, Bid -/- n=7, DKO n=8, TKO n=7. (F) RBCs (10^6/µl), (G) Hemoglobin (g/dL), and (H) Hematocrit (%) measured in WT n=6, TKO n=6, and TKORIPKD (+/-) n=6 mice. Note that TKORIPKD mice are heterozygous for RIPKD (+/-). (I) Number of cells in the bone marrow from WT n=7, TKO n=7, and TKORIPKD n=8 mice. (J) Myeloid progenitor cells (%) from lineage depleted WT n=12, TKO n=14, and TKORIPKD n=8 mice. (K) BrDU+ myeloid progenitors (%) from WT n=13, TKO n=15, and TKORIPKD n=8 mice. ns= not significant, *p<0.05 **p<0.01, ***p<0.001, and ****p<0.0001. Data are representative of mean +/- SEM.

Figure 5 : TKO bone marrow can reconstitute and outcompete Bid +/+ bone marrow but is unable to maintain long-term hematopoiesis.

(A) Percent CD45.2+ cells in Bid +/+, DKO, and TKO transplant mice at 8,12,16, and 20 weeks after transplantation. Mice were transplanted with experimental and control bone marrow at a 1:1 ratio. Bid +/+ n=7 DKO n=7 TKO n=6. Statistics demonstrate differences between Bid +/+ and TKO animals. (B) RBCs (10^6/µl) in transplanted Bid +/+, DKO, and TKO mice at 8, 12, 16, and 20 weeks after transplantation. Bid +/+ n=5 DKO n=8 TKO n=8. Statistics demonstrate differences between Bid +/+ and TKO animals. (C) Platelet counts (10^3/µl) in transplanted Bid +/+, DKO, and TKO mice at 8, 12, 16, and 20 weeks after transplantation. Bid +/+ n=5 DKO n=8 TKO n=8. Statistics demonstrate differences between Bid +/+ and TKO animals. (D) Mean platelet volume (FL) in transplanted Bid +/+, DKO, and TKO mice at 8, 12, 16, and 20 weeks post transplantation. Bid +/+ n=5 DKO n=8 TKO n=8. Statistics demonstrate differences between Bid +/+ and TKO animals. (E) Distribution of myeloid progenitor, LSK, and SLAM-HSC

populations in Bid +/+, DKO, and TKO transplanted mice. Bid +/+ n=5 DKO n=7 TKO n=6. (F) CD45.2 positive LSK cells in transplanted mice. (G) Secondary transplantation of DKO and TKO bone marrow (primary transplant in a 1:1 ratio with Bid +/+) to rigorously test HSC function. DKO n=8 TKO n=7. ns= not significant, *p<0.05 **p<0.01, ***p<0.001, and ****p<0.0001. Data are representative of mean +/- SEM.

Figure 6: TKO mice have increased TNFα which can be rescued by a genetic cross with RIPKD mice or the anti-inflammatory drug Enbrel.

(A) Fluorescent immunohistochemistry of WT, DKO, and TKO lungs stained for TNFα. Scale bar=50 microns. Experiment was completed two independent times. (B) Intracellular cytokine staining of bone marrow following 5 hours of LPS stimulation (200 ng/mL) + Golgi Plug. (C) TNFα (C), IL-6 (D), and IL-1β (E) positivity in myeloid progenitor and LSK populations in Bid+/+, DKO, and TKO mice treated as in (B). (F) Myeloid progenitor populations (left) and BrDU+ myeloid progenitors (right) in Bid +/+ and TKO mice before and after treatment with Enbrel (TNF decoy receptor). (G) RBCs (10^6/μl) (left) and platelet counts (10^3/μl) (right) in Bid +/+ and TKO mice. Bid +/+ n=4 TKO n=4. ns= not significant, *p<0.05, ***p<0.001, and ****p<0.0001. Data are representative of mean +/- SEM.

Figure 7: Human bone marrow failure in early Myelodysplastic syndrome (MDS) has a necrotic phenotype.

(A) Rip1 staining on paraffin-embedded human bone marrow aspirate for DAPI and Rip1. MDS subtypes include (from top to bottom): Refractory cytopenia with multilineage dysplasia (RCMD), Refractory anemia with excess blasts (RAEB-1), and Refractory anemia with ring sideroblasts (RARS). Scale bar=100 microns. Experiment was performed three independent times. (B) Cleaved caspase-3 staining on paraffin-embedded human bone marrow aspirate. Experiment was performed three independent

times. Scale bar=50 microns. (C) Phospho-MLKL staining on paraffin-embedded human bone marrow aspirate. Experiment was performed two independent times. Scale bar=50 microns. (D) Table demonstrating positivity of human samples for Rip1 and phospho-MLKL by subtype. (E) Transmission electron microscopy (TEM) of human bone marrow aspirate following RBC lysis from a normal control donor, an early MDS and RAEB patient. Scale bar =2 microns, 4,400X magnification. Quantitation of apoptotic or necroptotic cell death for each patient represented in graph (right).

Figure 1

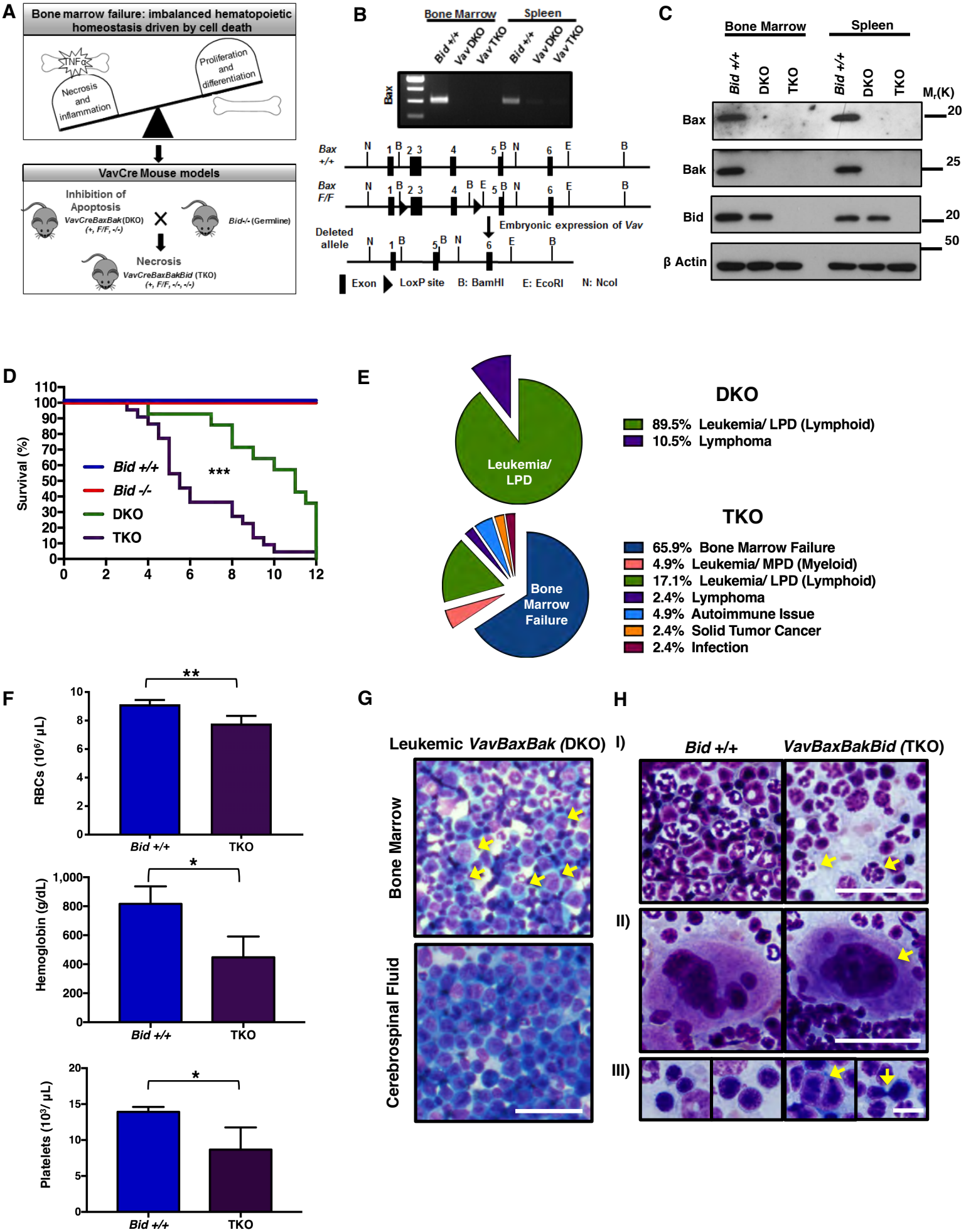

A
Bone marrow failure: imbalanced hematopoietic homeostasis driven by cell death
TNFα
Necrosis and inflammation
Proliferation and differentiation
VavCre Mouse models
Inhibition of Apoptosis
VavCreBaxBak (DKO)
(+, F/F, -/-)
Bid-/- (Germline)
Necrosis
VavCreBaxBakBid (TKO)
(+, F/F, -/-, -/-)
B
Bone Marrow
Spleen
Bid +/+
Vav DKO
Vav TKO
Bax
Bax +/+
Bax F/F
Deleted allele
Embryonic expression of Vav
Exon
LoxP site
B: BamHI
E: EcoRI
N: NcoI
C
Bone Marrow
Spleen
Bid +/+
DKO
TKO
Mr(K)
Bax
Bak
Bid
β Actin
20
25
20
50
D
Survival (%)
Bid +/+
Bid -/-
DKO
TKO
***
E
Leukemia/ LPD
Bone Marrow Failure
DKO
89.5% Leukemia/ LPD (Lymphoid)
10.5% Lymphoma
TKO
65.9% Bone Marrow Failure
4.9% Leukemia/ MPD (Myeloid)
17.1% Leukemia/ LPD (Lymphoid)
2.4% Lymphoma
4.9% Autoimmune Issue
2.4% Solid Tumor Cancer
2.4% Infection
F
RBCs (10⁶/ µL)
Hemoglobin (g/dL)
Platelets (10³/ µL)
Bid +/+
TKO
**
*
*
G
Leukemic VavBaxBak (DKO)
Bone Marrow
Cerebrospinal Fluid
H
I)
II)
III)
Bid +/+
VavBaxBakBid (TKO)

Figure 2

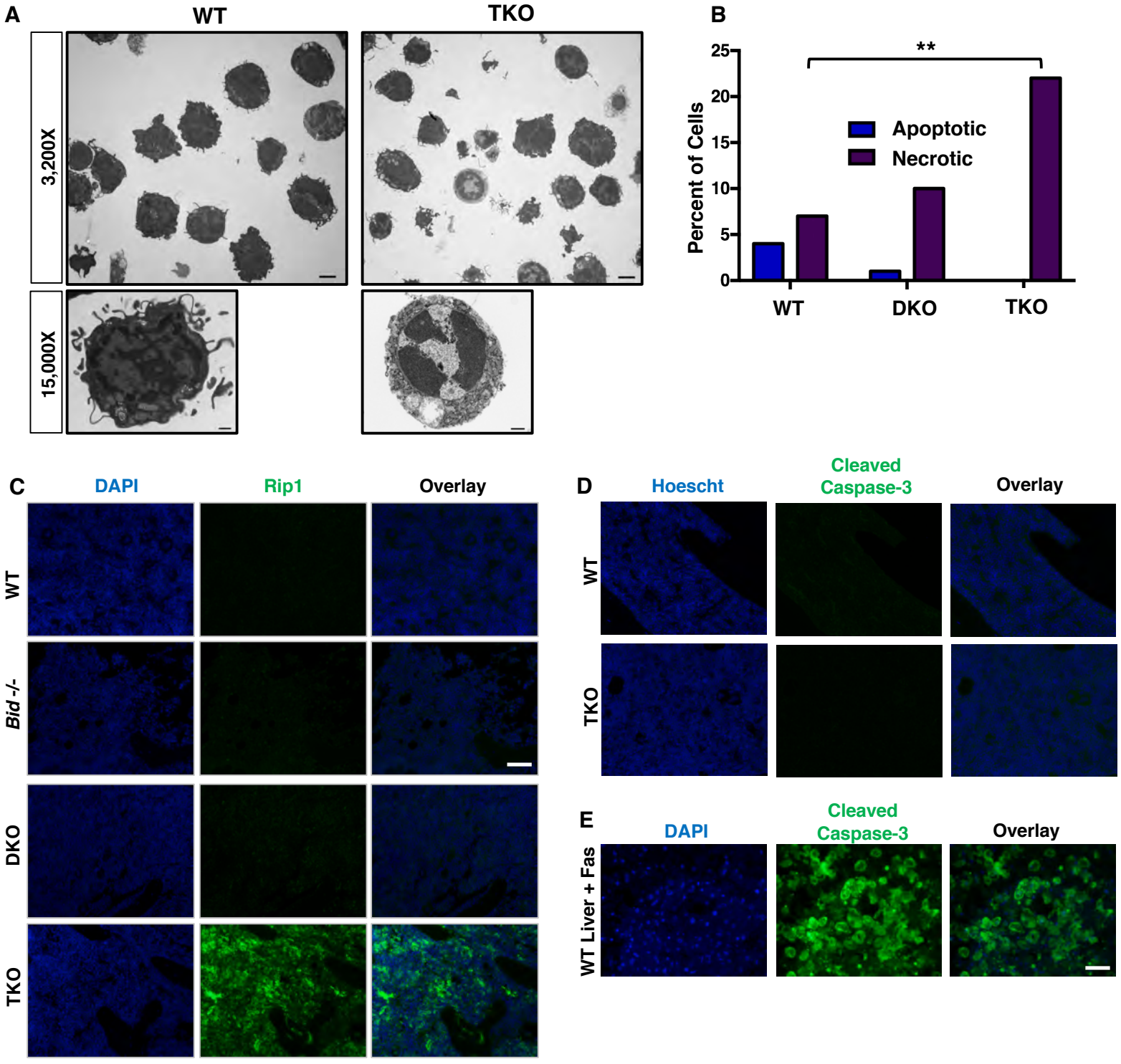

A
WT
TKO
3,200X
15,000X
B
**
Percent of Cells
25
20
15
10
5
0
Apoptotic
Necrotic
WT
DKO
TKO
C
DAPI
Rip1
Overlay
WT
Bid -/-
DKO
TKO
D
Hoescht
Cleaved Caspase-3
Overlay
WT
TKO
E
DAPI
Cleaved Caspase-3
Overlay
WT Liver + Fas

Figure 3

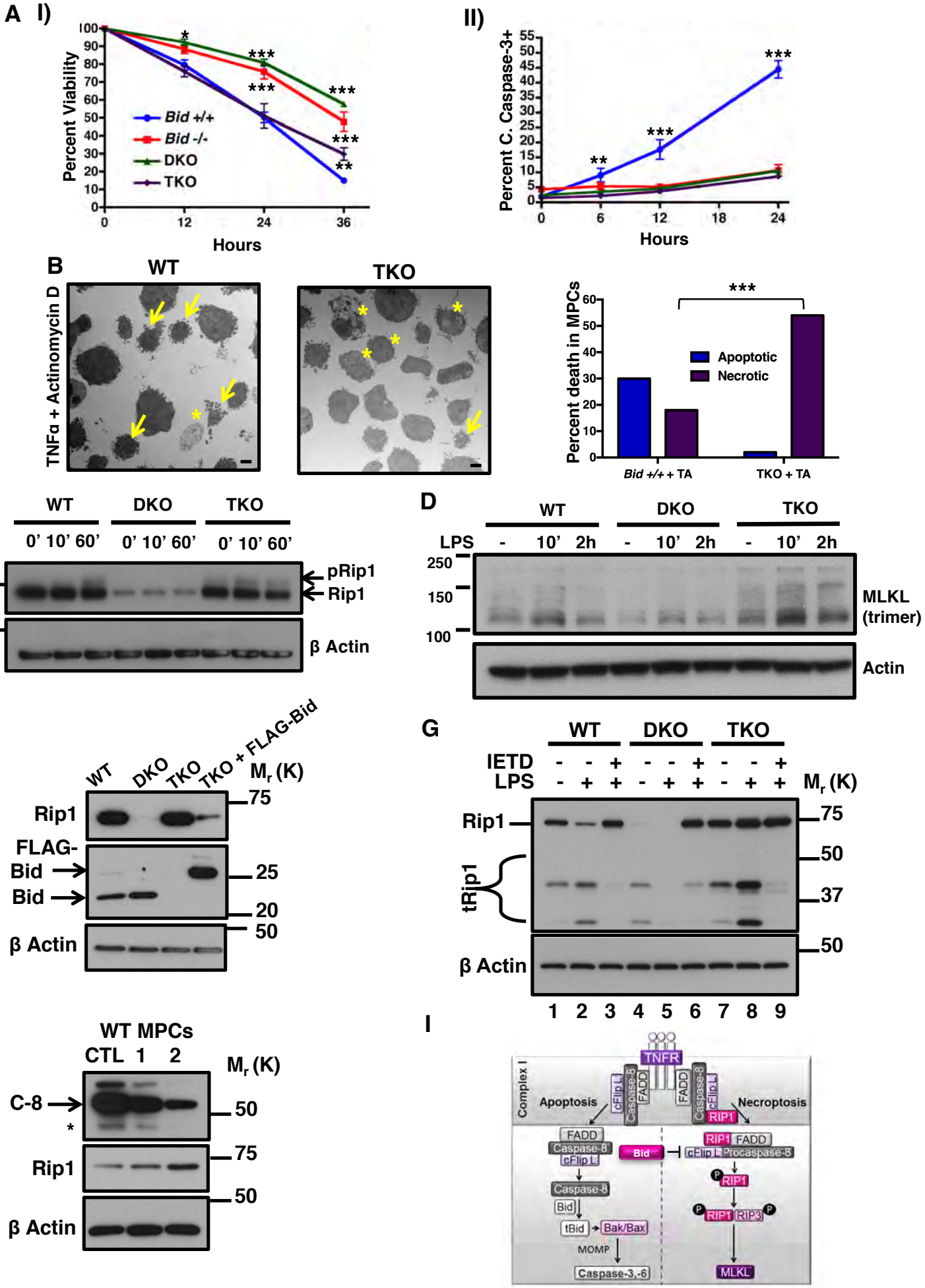

A I)
Percent Viability
Hours
Bid +/+
Bid -/-
DKO
TKO
II)
Percent C. Caspase-3+
Hours
B
WT
TKO
TNFα + Actinomycin D
Percent death in MPCs
Apoptotic
Necrotic
Bid +/+ + TA
TKO + TA
C
WT
DKO
TKO
LPS (mins)
0' 10' 60'
Mr(K)
75
50
pRip1
Rip1
β Actin
D
WT
DKO
TKO
LPS
- 10' 2h
250
150
100
MLKL (trimer)
Actin
F
WT
DKO
TKO
TKO + FLAG-Bid
Mr (K)
Rip1
FLAG-Bid
Bid
β Actin
75
25
20
50
G
WT
DKO
TKO
IETD
LPS
Mr (K)
Rip1
tRip1
β Actin
75
50
37
50
1 2 3 4 5 6 7 8 9
H
WT MPCs
CTL 1 2
Mr (K)
C-8
*
Rip1
β Actin
50
75
50
I
Complex I
TNFR
Apoptosis
Necroptosis
FADD
Caspase-8
cFlip L
RIP1
Bid
Procaspase-8
tBid
Bak/Bax
MOMP
Caspase-3,-6
RIP3
MLKL

Figure 4

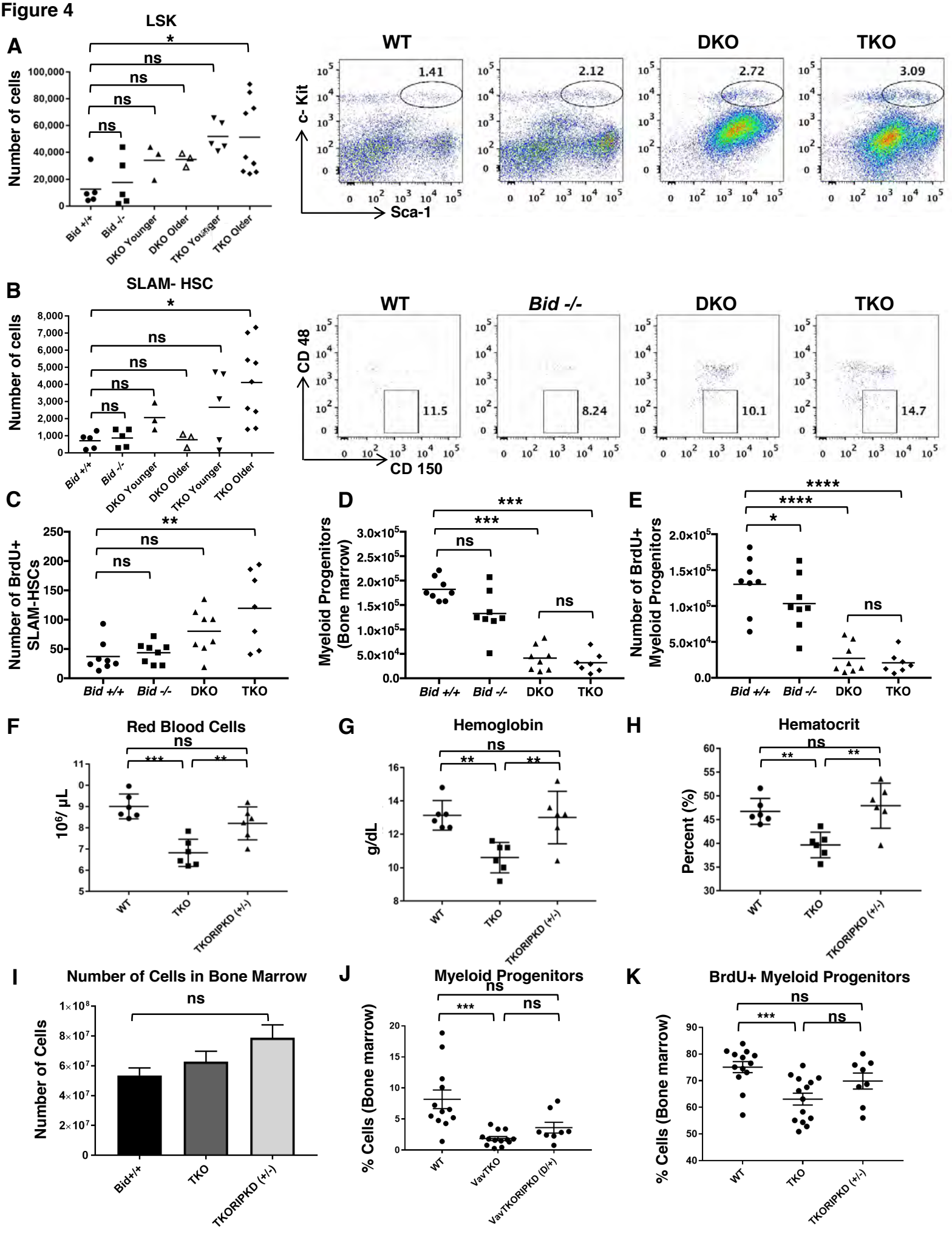

A
LSK
Number of cells
Bid +/+
Bid -/-
DKO Younger
DKO Older
TKO Younger
TKO Older
WT
DKO
TKO
c- Kit
Sca-1
1.41
2.12
2.72
3.09
B
SLAM- HSC
Number of cells
WT
Bid -/-
DKO
TKO
CD 48
CD 150
11.5
8.24
10.1
14.7
C
Number of BrdU+ SLAM-HSCs
D
Myeloid Progenitors (Bone marrow)
E
Number of BrdU+ Myeloid Progenitors
F
Red Blood Cells
10⁶/ µL
TKORIPKD (+/-)
G
Hemoglobin
g/dL
H
Hematocrit
Percent (%)
I
Number of Cells in Bone Marrow
Number of Cells
Bid+/+
TKO
TKORIPKD (+/-)
J
Myeloid Progenitors
% Cells (Bone marrow)
VavTKO
VavTKORIPKD (D/+)
K
BrdU+ Myeloid Progenitors
% Cells (Bone marrow)

Figure 5

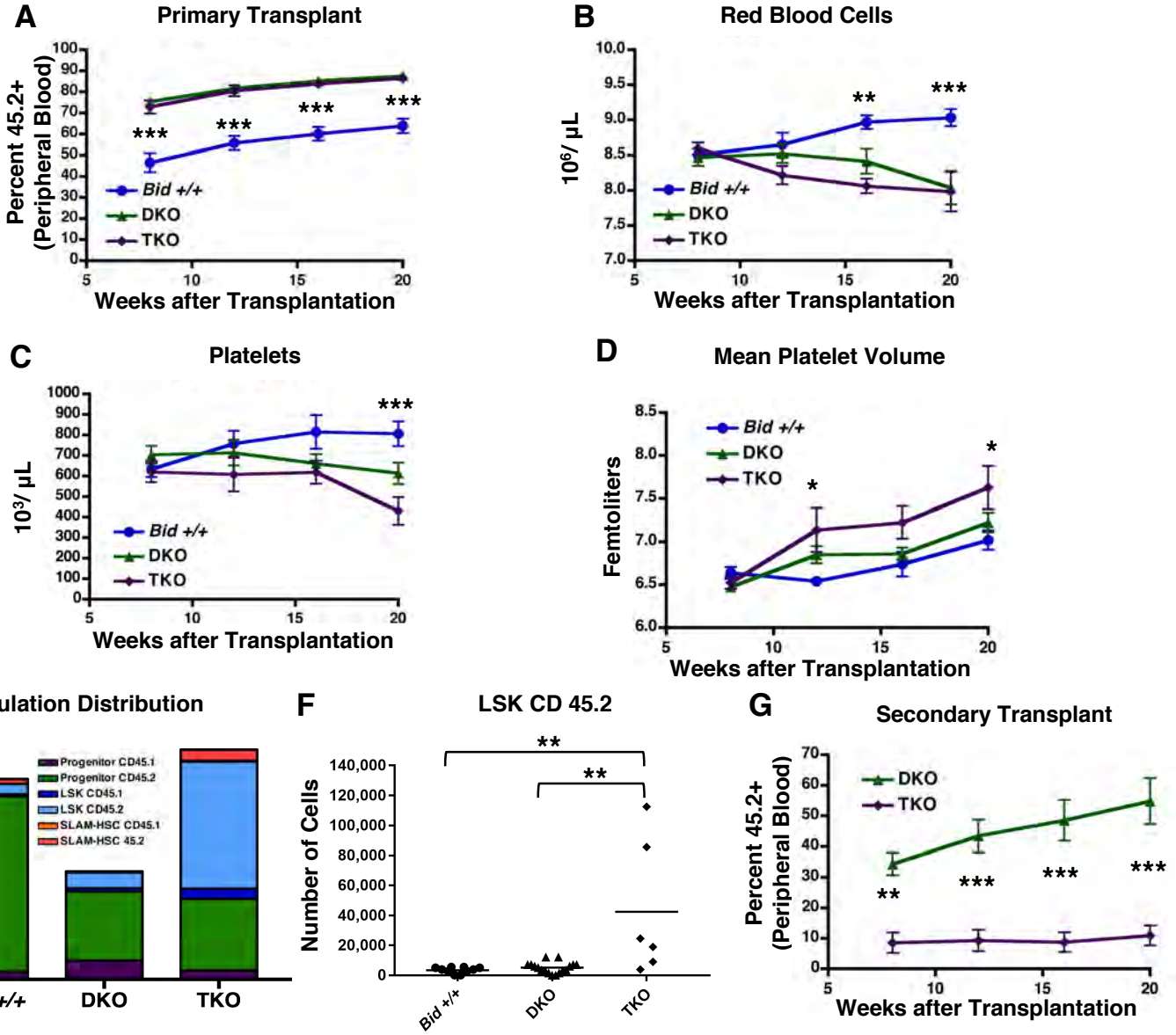

A
Primary Transplant
Percent 45.2+ (Peripheral Blood)
Weeks after Transplantation
Bid +/+
DKO
TKO
B
Red Blood Cells
10⁶/ µL
C
Platelets
10³/ µL
D
Mean Platelet Volume
Femtoliters
E
Population Distribution
Avg. Number of Cells
Progenitor CD45.1
Progenitor CD45.2
LSK CD45.1
LSK CD45.2
SLAM-HSC CD45.1
SLAM-HSC 45.2
F
LSK CD 45.2
Number of Cells
G
Secondary Transplant

Figure 6

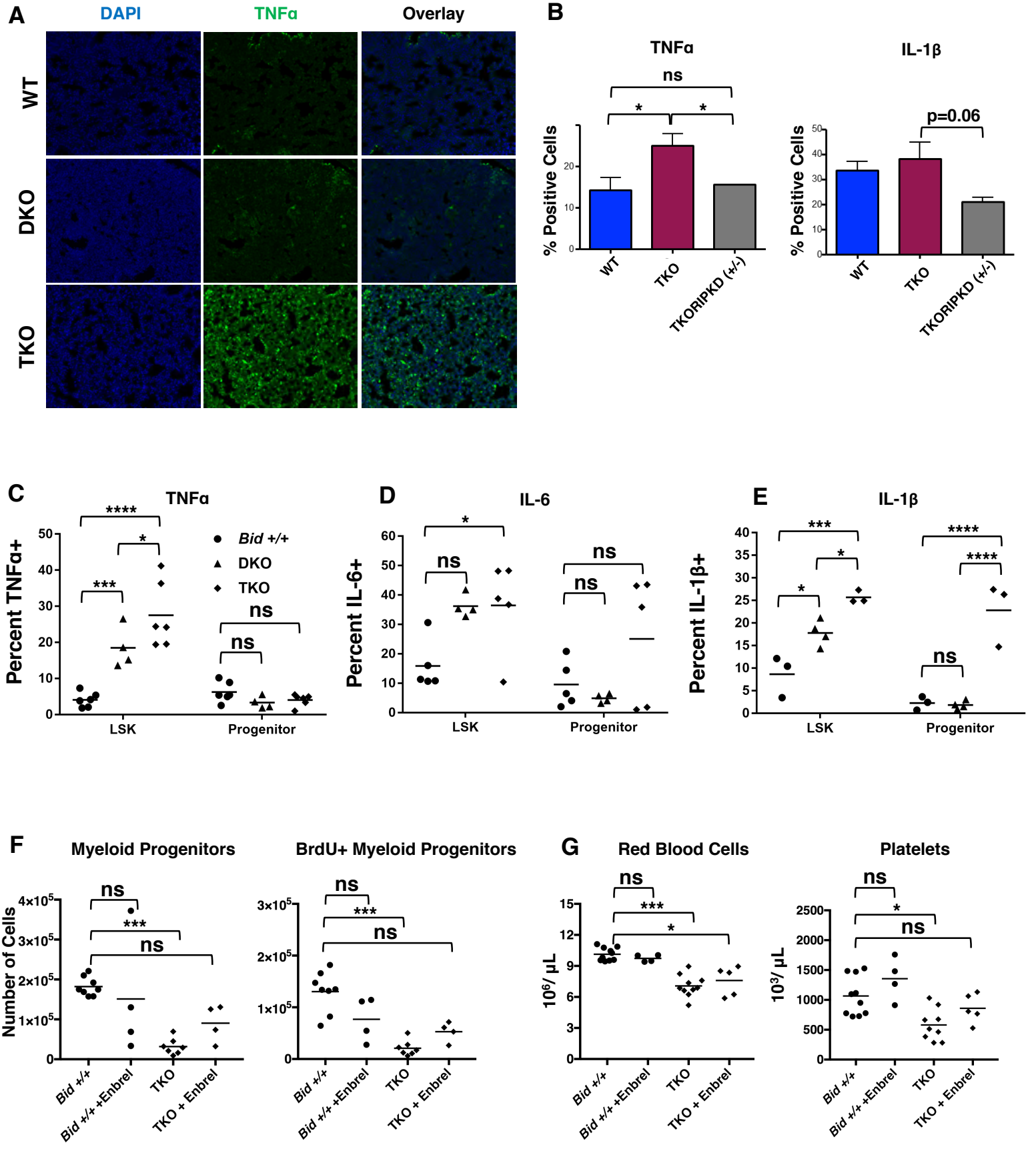
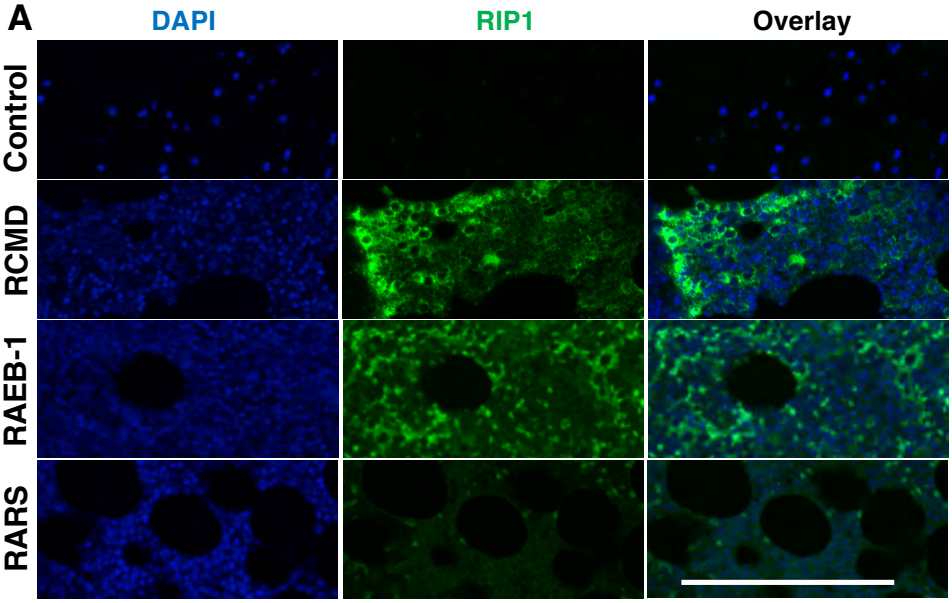
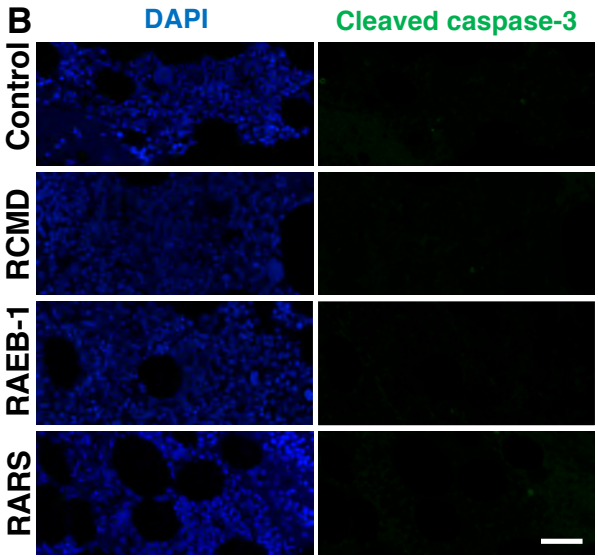
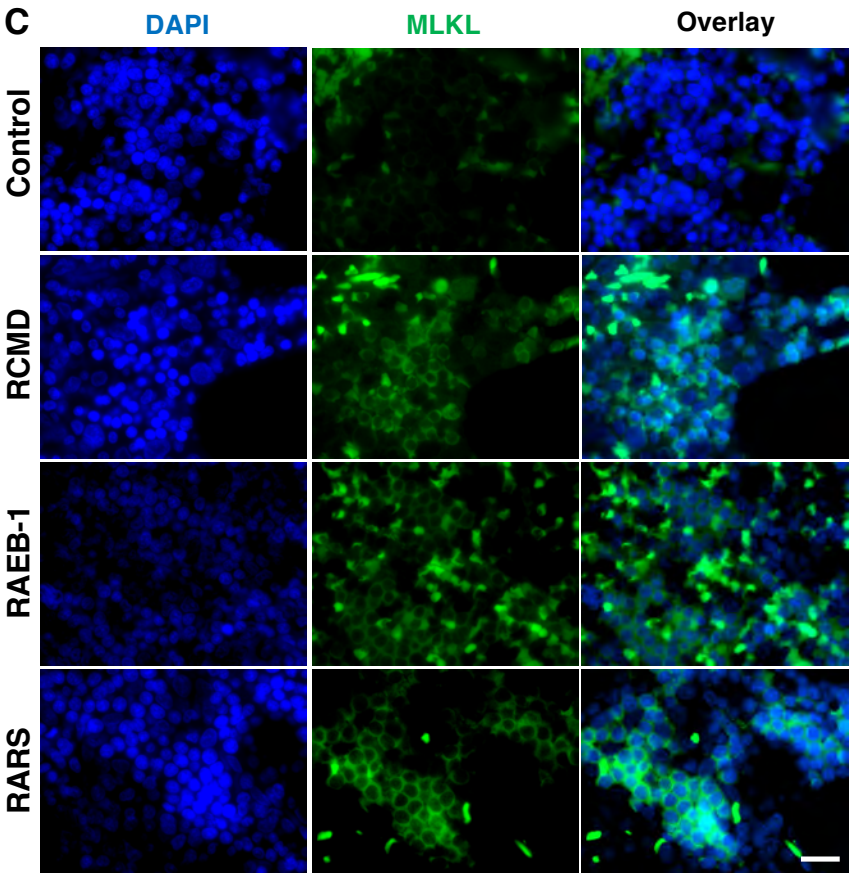

A
DAPI
TNFα
Overlay
WT
DKO
TKO
B
TNFα
IL-1β
% Positive Cells
ns
p=0.06
WT
TKO
TKORIPKD (+/-)
C
TNFα
Percent TNFα+
Bid +/+
DKO
TKO
LSK
Progenitor
D
IL-6
Percent IL-6+
E
IL-1β
Percent IL-1β+
F
Myeloid Progenitors
BrdU+ Myeloid Progenitors
Number of Cells
Bid +/+
Bid +/+ +Enbrel
TKO
TKO + Enbrel
G
Red Blood Cells
Platelets
10^6/ µL
10^3/ µL

Figure 7







D % Positivity/Total Samples

Marker	Control	RCMD	RAEB-1/2	RARS
Rip1	1/4	6/6	3/7	1/1
pMLKL	1/4	5/5	5/7	1/1